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A simplified competitive protein-binding assay for 25-Hydroxycalciferol

(ESSAI SIMPLIFIE COMPETITIF PAR PROTEINE VECTRICE
POUR 25-HYDROXYCALCIFEROL)

THESE

présentée à la Faculté de médecine de l'Université de Genève
pour obtenir le grade de docteur en médecine

par

Bernardo GARCIA-PASCUAL

de

MADRID (Espagne)

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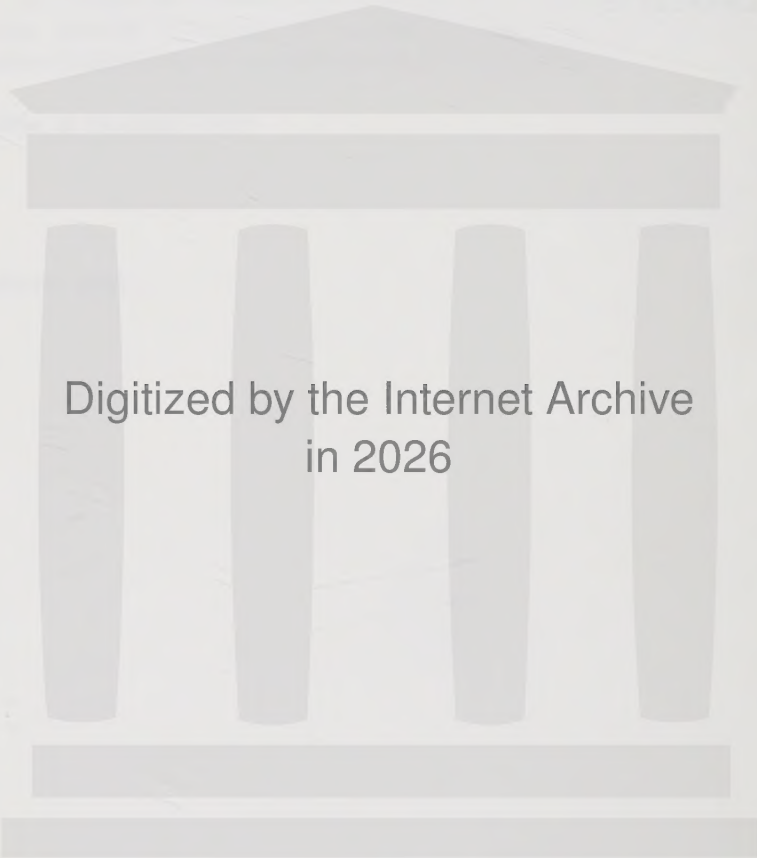
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A SIMPLIFIED COMPETITIVE PROTEIN-BINDING ASSAY FOR 25-HYDROXYCALCIFEROL

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Summary

A single and reliable, competitive, protein-binding assay for plasma 25-hydroxycalciferol (25-OH-D), based on a previously described method which omits the chromatographic step, has been tested without the use of beta-lipoproteins. The accuracy is similar in the absence of beta-lipoproteins and in their presence, the recoveries being respectively 110–136% and 74–85%. The intra-assay coefficient of variation is 7.23% in the absence versus 12.56% in the presence of beta-lipoproteins. The sensitivity of the described assay is 0.01 ng per assay tube and 1 $\mu\text{g/l}$ plasma. The mean plasma 25-OH-D level in eighteen normal Swiss volunteers aged 20 to 40 years is $39.5 \pm \text{S.D. } 9.3 \mu\text{g/l}$. This level is similar to those found in Western Europe and U.S.A. subjects. Ten alcoholic patients without cirrhosis have significantly lower 25-OH-D plasma levels ($13.0 \pm \text{S.D. } 8.9 \mu\text{g/l}$) than appropriate age controls. This precise and accurate method is convenient for clinical studies since both chromatographic and beta-lipoproteins steps are avoided.

Introduction

A number of competitive protein-binding assays for 25-hydroxycalciferol (25-OH-D) have been described in the last few years [1–4]. The source of the specific binding protein used in the assays has been either rat serum [1,3,4] or rat kidney cytosol [2] and the assays have involved chromatography of the plasma lipid extracts. This was felt to be necessary to remove non-specific binding substances as well as other metabolites of vitamin D known to bind to these proteins. Recently, an alternative assay has been described [5] in which the chromatographic step has been omitted, but unfortunately little information was provided on the precision and accuracy of this much simpler method. Dif-

difficulties which we experienced with the method were eventually traced to the beta-lipoproteins which are used primarily to effect complete solubilisation of the 25-OH-D extract. But as the use of beta-lipoproteins in assays for 25-OH-D is unique to the methods described by Belsey et al. [1,5] their inclusion would not seem to be essential. The present paper reports our experience with a competitive protein-binding assay for the estimation of 25-OH-D in lipid extracts of human plasma without chromatographic purification and without beta-lipoproteins.

Experimental

Materials

[26,27- Me - 3H]-25-Hydroxycholecalciferol, specific activity 6.9 Ci/mmol, was purchased from the Radiochemical Centre, Amersham, Bucks., U.K., and diluted as appropriate with ethanol after removing the benzene in which it was originally dissolved. Crystalline 25-hydroxycholecalciferol (25-OH- D_3) was obtained from Philips Duphar, Amsterdam, Netherlands, and diluted in ethanol to provide a series of standard solutions ranging from 12 to 0.3 μg 25-OH- D_3 per litre. Each set of standards was used on not more than six occasions and stored at 4°C.

Charcoal (Norit GSX) was obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K. Dextran of mol. wt. 60 000–90 000, was obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K. The charcoal (5.0 g) was coated with 0.5 g of Dextran in 200 ml of one of the two buffers used in these studies. For use in the assay this solution was further diluted, 1 : 5 (v/v), with the same buffer.

Buffers

BAB. A stock solution of barbital/acetate buffer (76.3 mmol/l sodium barbital, 118.5 mmol/l sodium acetate) designated BAB was prepared. The working solution was made by diluting 50 ml of the stock solution with 850 ml 9% NaCl, adjusting with 1 mol/l HCl to pH 8.6 and adding distilled water to give a total volume of 1 litre.

BALPB. Beta-lipoproteins were obtained from four different human plasma samples by precipitation with heparin and $MnCl_2$ as previously described [1]. This beta-lipoprotein solution was further diluted 1/200 with BAB for use in the assay.

Serum binding proteins

Piebald weanling rats were raised on a vitamin D-deficient diet as described by Numerof et al. [6]. After 2–3 weeks, blood was collected by intracardial puncture and the serum diluted with the buffers to obtain 50% binding of the tritiated 25-OH- D_3 .

Scintillator

5.0 g 2,5-diphenyloxazole (PPO) and 0.3 g of 1,4-bis-(5-phenyloxazolyl)-2) (POPOP) were dissolved in 1000 ml of toluene and 500 ml of Triton X-100.

Assay procedure

The competitive protein-binding assay for 25-OH-D was essentially that described by Belsey et al. [5] Plasma or serum (100 μ l) was pipetted into tubes containing 900 μ l of ethanol with 4000 dpm of radioactive 25-OH-D₃, the solutions were then thoroughly mixed and left at 4°C for 30 min. The precipitate was sedimented by centrifugation at 2000 $\times g$ for 30 min at 4°C and the supernatant removed. The recovery of 25-OH-D₃ from the plasma was assessed by measuring the radioactivity in a 100- μ l aliquot of the supernatant. The average recovery from 63 such extractions was $91.0 \pm \text{S.D. } 1.6\%$. Aliquots (100 μ l) of the supernatant were also pipetted into each of three tubes containing 900 μ l of the diluted serum binding-protein and, to assess non-specific binding, a further 100- μ l aliquot of the supernatant was added to 900 μ l of the appropriate buffer. For each assay a standard curve was prepared by adding in triplicate an aliquot of each of the set of standards to the diluted serum binding protein and a further aliquot to the buffers alone. All solutions were then mixed and left at 4°C for 2 h.

Separation of the 25-OH-D bound to the serum binding-protein from the unbound fraction was carried out by leaving the solutions for 15 min at 4°C with 0.4 ml of the dextran-coated charcoal solution. The solutions were then centrifuged for 10 min at 2000 $\times g$ and the radioactivity i.e. bound 25-OH-D, measured in aliquots of the supernatant. After correcting for the non-specific binding, the bound radioactivity (i.e. complex of radioactive 25-OH-D₃ and serum binding protein) was expressed as a percentage of the radioactivity originally incubated with the binding proteins.

The calibration curve (Fig. 1) was constructed by plotting the percentage of

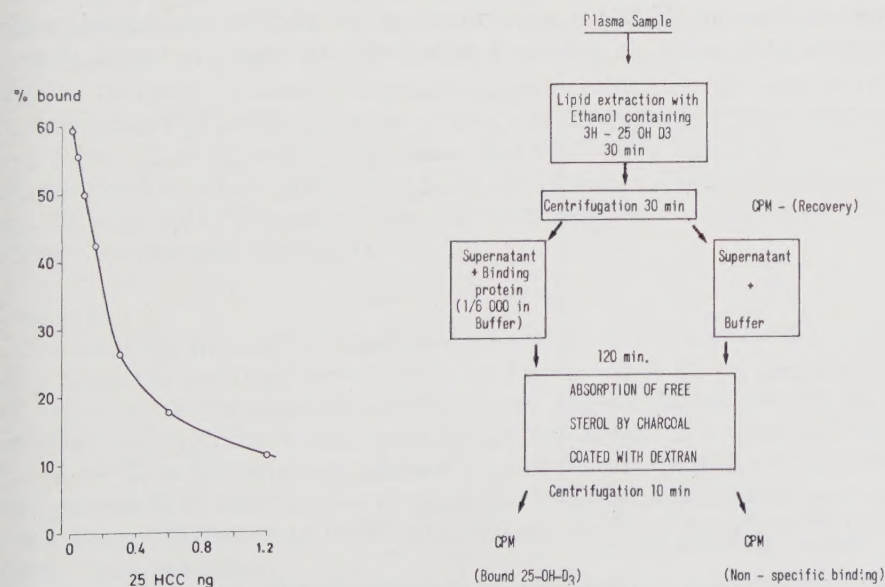


Fig. 1. A typical standard curve for 25-hydroxycalciferol assay.

Fig. 2. Outline of assay method.

radioactivity bound against the amount of non-radioactive steroid. Unknown amounts of 25-OH-D were determined by reference to the calibration curve and appropriate corrections were made for the aliquots of plasma used and for the recovery from the extraction step. An outline of the assay is illustrated in Fig. 2.

Subjects

Thirty five healthy Swiss volunteers were divided into three separate groups. Eighteen, aged 20 to 40 (9 males and 9 females); seven aged 40 to 60 (4 males and 3 females) and ten geriatric subjects aged 60 to 80 (4 males and 6 females).

Three osteomalacic patients with bone pain, hypocalcemia, hypophosphatemia, hypocalcemia and high levels of alkaline phosphatase, were studied. None of these patients had radiologic signs of vitamin D deficiency. In two, the bone histology was typical of osteomalacia.

Plasma samples were also collected from ten alcoholic patients without clinical and biochemical signs of cirrhosis. All samples were obtained between January and April 1975.

Results

Accuracy

Increasing amounts of unlabelled 25-OH-D₃ were added to aliquots of pooled plasma and assayed three times in triplicate. Using the BAB buffer in the assay average recoveries of 25-OH-D₃ of 110–136% were obtained over the range of 9–36 $\mu\text{g/l}$ of added steroid (Fig. 3), whereas using the BALPB buffer resulted in recoveries of 74–85% over the same range of added unlabelled 25-OH-D₃. The endogenous level of 25-OH-D determined at no addition of steroid, was subtracted from each value. In a second experiment to assess accuracy, unla-

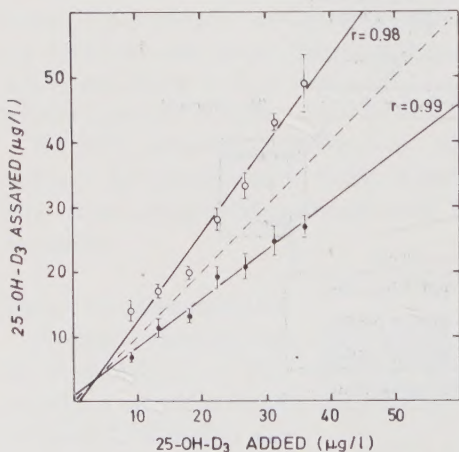


Fig. 3. Accuracy of 25-OH-D assay. Increasing amounts of unlabelled 25-OH-D₃ were added to a plasma sample and the assay carried out as described under Experimental. Each point represents the mean of three assays in triplicate $\pm$ S.E.M. A correction has been applied to each point for the endogenous concentration of 25-OH-D in the plasma sample, 25.9 $\mu\text{g/l}$ with BALPB (●) and 29.6 $\mu\text{g/l}$ with BAB (○).

TABLE I

RECOVERY OF ADDED 25-OH-D₃ IN DIFFERENT PLASMA SAMPLES

The 25-OH-D₃ concentration in eight different plasma samples was assayed (A) and the measurement repeated after the addition of 26.3 ng to 1 ml of each plasma sample (B). The recovery of the added 25-OH-D₃ as measured by this assay was obtained by difference of these two measurements. Results are expressed as μg of 25-OH-D₃/ μg /l of plasma.

Sample No.	A	B	B - A
1	9.6	37.4	27.8
2	18.2	52.4	34.2
3	23.5	49.2	25.7
4	29.9	56.7	26.8
5	31.0	64.2	33.2
6	44.9	85.6	40.7
7	48.1	74.9	26.8
8	101.7	141.3	39.6

belled 25-OH-D₃ (26.3 ng) was added to aliquots (1 ml) of eight different plasma samples containing endogenous concentrations of 25-OH-D ranging from 9.6 to 101.7 μg /l. The samples were then assayed in BAB buffer. After subtracting the 25-OH-D endogenous concentration of each sample, the mean recovery of added 25-OH-D₃ was found to be 120% (31.8 ng 25-OH-D/ml) and the coefficient of variation 18.8%.

Precision of assay

Ten aliquots from a single plasma sample were extracted separately and assayed using BAB buffer. The mean concentration was found to be $44.2 \pm \text{S.D. } 3.2$ μg /l and the coefficient of variation was 7.23%. When the same experiment was repeated with BALPB buffer in the assay procedure the mean 25-OH-D concentration was $38.6 \pm \text{S.D. } 4.8$ μg /l and the coefficient of variation was 12.56%. In order to study interassay variation which occurs with BAB buffer, six estimations in duplicate were carried out on aliquots from a pooled plasma over a period of 6 weeks. The mean 25-OH-D value was $34.6 \pm \text{S.D. } 5.2$ μg /l and the coefficient of variation was 15.1% ($n = 10$). The same plasma aliquots assayed with BALPB buffer showed a mean of $25.5 \pm \text{S.D. } 4.4$ μg /l and the coefficient of variation was 17.31%.

Sensitivity

The smallest amount of unlabelled 25-OH-D₃ which can be detected in this assay from the standard curve shown in Fig. 1, was 0.01 ng per tube (10% displacement of the labelled steroid) or 1 μg /l plasma. Displacements of 50% occur with 0.25 ng/tube. It has been found that levels of 1–100 μg /l of 25-OH-D₃ can be detected routinely using 0.1 ml of plasma. Samples of distilled water were processed to evaluate the method blank but no displacement was obtained in the assay. Plasma from three osteomalacic patients yielded a 25-OH-D level of $4.6 \pm \text{S.D. } 0.47$ μg /l.

Studies in normal and alcoholic plasmas

In plasma from a group of 18 Swiss volunteers, aged 20–40 years, on a diet

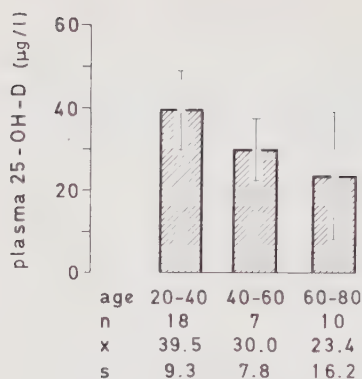


Fig. 4. Plasma 25-OH-D in normal persons divided into 3 groups according to their age. n = number of volunteers; x = mean levels ($\mu\text{g/l}$); s = standard deviation.

unrestricted with regard to vitamin D, the circulating 25-OH-D levels were found to be $39.5 \pm \text{S.D. } 9.3 \mu\text{g/l}$ with a range of $25.6\text{--}61.1 \mu\text{g/l}$ (Fig. 4). Seven further volunteers aged 40–60 years had an average plasma 25-OH-D level of $30.4 \pm 7.8 \mu\text{g/l}$ with a range of $19.7\text{--}39.5 \mu\text{g/l}$. In a group of ten people over 60 years old, the mean concentration of plasma 25-OH-D was $23.4 \pm 16.2 \mu\text{g/l}$ with a range of $6.6\text{--}54.9 \mu\text{g/l}$. This concentration is significantly lower than the 20–40 year old group ($P < 0.01$).

In 10 alcoholic patients without cirrhosis aged from 37–56 years and on an unrestricted vitamin D intake the plasma 25-OH-D levels were found to be $13.0 \pm 8.9 \mu\text{g/l}$. This level of plasma 25-OH-D is significantly lower than the level found in an appropriate age-control group ($P < 0.01$).

Discussion

The results reported in this paper confirm the validity of plasma 25-OH-D measurements by competitive protein-binding assay without preparative chromatography. The use of human beta-lipoproteins in the buffer as recommended by Belsey et al. [5] resulted in variable replacement of the radioactive 25-OH-D₃ from the binding sites on the serum proteins. It was also found that lipoprotein preparations from different individuals were not equivalent as assessed by the extent of the replacement of radioactive 25-OH-D₃. However, it is apparent from the results presented here that a simple, reliable and sensitive assay for plasma 25-OH-D levels not involving a chromatographic purification step is possible without the addition of lipoproteins to the incubation buffer. The sensitivity of the assay for 25-OH-D is more than sufficient for most clinical and epidemiological purposes since 10 pg/tube ($1 \mu\text{g/l}$ plasma) can be detected and the accuracy of the method is also adequate for these purposes. From Fig. 3 it can be seen that the inclusion of lipoproteins in the assay results in a tendency to underestimate 25-OH-D₃ levels, presumably as the steroid remains on the lipoproteins rather than becoming bound to the specific carrier protein. On the other hand this present method tends to overestimate the levels but this is not unexpected as vitamin D and metabolites other than 25-OH-D show some com-

petition with 25-OH-D for the specific binding sites (Lawson, unpublished observations). Consequently any assay for 25-OH-D not involving chromatography must estimate metabolites such as 24,25-(OH)₂D₃ and 25,26-(OH)₂D₃. In this connection it was significant that the plasma samples from patients with biochemical evidence of osteomalacia had very low levels of 25-OH-D (i.e. 4.6 µg/l). Previous investigators using an assay involving chromatography have found that rickets and osteomalacia due to a nutritional deficiency is always associated with plasma 25-OH-D levels of not more than 4 µg/l [7].

In this study the mean plasma 25-OH-D level of normal Swiss volunteers, aged 20–40 years, was $39.5 \pm \text{S.D. } 9.3 \text{ µg/l}$. These plasma samples were obtained during late winter months. The level is similar to that reported for undated plasma samples from normal German volunteers [8], $37.8 \pm \text{S.D. } 16.5 \text{ µg/l}$. The values reported for the 25-OH-D levels in plasma of American volunteers cover a wider range, e.g. Haddad and Chyu [2] quote levels of 27.3 µg/l , while more recently the same group [9] found levels of $23.6 \pm \text{S.D. } 6.4 \text{ µg/l}$ for plasma samples obtained from 51 patients during February–April. Belsey et al. [5] found 25-OH-D levels in undated plasma samples from fifteen volunteers of $35.2 \pm \text{S.D. } 13.9 \text{ µg/l}$. On the other hand there are several reports that 25-OH-D levels in plasma samples from British subjects range from 22.6 µg/l in the late summer months [10] (when the level is highest), to less than 13 µg/l in the winter period [3,4,10].

There is therefore accumulating evidence that when allowance is made for the time of the year at which the plasma sample is taken, the plasma 25-OH-D level in British subjects is considerably lower than that found in Western Europe and the U.S.A. Consequently consideration should be given to the question of whether this lower level of 25-OH-D in British people contributes to the aetiology of metabolic bone disease and related disorders of calcium metabolism as these diseases appear in Britain.

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